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cAMP inhibits induction of interleukin 2 but not of interleukin 4 in T cells

(T helper cell subsets/signaling/inositol phospholipid pathway/transcriptional control/chloramphenicol acetyltransferase)

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ABSTRACT **In this report, we explore the nature of the inductive stimuli leading to expression of the divergently regulated lymphokines interleukin 2 (IL-2) and interleukin 4 (IL-4). Elevation of cAMP levels blocks IL-2 induction while sparing IL-4 induction. These effects are gene-specific, not cell-specific, and can be observed in the same cells. Transient transfection experiments using murine IL-2 regulatory sequences to drive expression of a reporter gene show at least part of the inhibition to act at the transcriptional level. The possible biological significance of these results is indicated by the observation that representative type 2 helper T-cell lines maintain significantly higher levels of cAMP per cell than a type 1 helper T-cell line. Fresh splenic CD4$^+$ T cells, which preferentially make IL-2, have particularly low levels of cAMP per cell and a low capacity to elevate cAMP in response to forskolin. However, their response to forskolin increases significantly after several days of stimulation. These results suggest a potential link between differential cAMP regulation and the divergence of memory T cells into effector subsets.**

Subsets of T cells may be defined by the way they utilize similar stimuli to effect distinct outcomes at the transcriptional level. For example, activated type 1 helper T-cell (T_H1) lines synthesize interleukin 2 (IL-2), interferon-γ, and lymphotoxin in preference to IL-4 and IL-5, which are the products of type 2 helper T (T_H2) cells (1). Both T_H subsets use CD4 as a coreceptor and recognize antigen in association with class II major histocompatibility complex molecules. A central question is at what level the mechanisms operate that restrict T cells to one or another response type. The induction of either type of gene expression response depends on two steps: (*i*) the coupling of membrane receptors to intracellular signaling pathways and (*ii*) the effect of the resulting signaling mediators on transcription factors and/or on chromatin structure and RNA stability. It is known that the frequency of IL-2 producers in various populations and the levels of IL-2 mRNA that they accumulate are a function of the particular activating stimulus used (2–4). This suggests that the ultimate response phenotype of a T cell may not be heritably fixed but may be influenced by the type of external stimulus it receives.

In fact, several recent reports suggest that upon repeated exposure to antigen, CD4$^+$ T cells *in vivo* do shift their response type. In certain cases, the population appears progressively to lose the ability to express IL-2 while enhancing its ability to make IL-4 (5–7). An important question is whether such T-cell populations are developmentally preprogrammed to differentiate from IL-2 producers to IL-4 producers or whether instead they may be altered by the particular signaling events induced by contact with antigen. Signaling modulation could either result in preferential activation of a preexisting T-cell subset or result in "instructive" modification of the gene batteries expressed by individual responding cells. The phenomenon of T_H1-specific induction of anergy (8–11) is an example of the former mechanism. Differences between T_H1 and T_H2 clones in the coupling of their T-cell receptors (TCR) to signaling cascades (12) reinforce this concept of preexisting distinctions. On the other hand, the tendency of rare clones producing both IL-2 and IL-4 to lose expression of IL-2 preferentially, either on extended passage (13) or on acute exposure to glucocorticoids (14), is an example of the latter.

This report demonstrates that the common second messenger cAMP may participate in shifts to T_H2-like responses in individual cells. Established T_H2 cells were found to maintain significantly higher levels of intracellular cAMP than T_H1 cells. When stimuli that bypass the TCR are used to induce both IL-2 and IL-4 in model systems, the presence of cAMP agonists selectively blocks IL-2 induction without affecting the induction of IL-4. The blockade of IL-2 synthesis appears to be exerted at least partly by gene-specific inhibition of transcription from the IL-2 promoter. Most strikingly, the differential effects of cAMP on IL-2 and IL-4 expression need not simply reflect differences among cellular subsets in susceptibility to inhibition, because they can be observed in the same cells.

MATERIALS AND METHODS

Drugs and Reagents. Stocks of the phorbol ester phorbol 12-myristate 13-acetate (PMA) and the calcium ionophore A23187 were made up in Me_2SO and used at final concentrations of 10 ng/ml for PMA and 37 ng/ml for A23187. The adenylate cyclase activator, forskolin, $N^6,O^{2'}$-dibutyryladenosine 3',5'-cyclic monophosphate (Bt_2cAMP), and prostaglandin E_1 (PGE_1) were purchased from Sigma and Calbiochem. In all cases the final organic solvent concentration was 0.1%.

Cell Lines. The T_H1 line A.E7 and the T_H2 lines D10.G4.1 and CDC-25 were passaged every 7–10 days with antigen and mitomycin C-treated feeder cells as described (15–17). The murine thymoma EL4.E1, the human T-leukemic line Jurkat, and the IL-3-dependent premast cell line 32Dcl5 were grown as described (18).

Plasmids and Transfections. Plasmids containing various amounts of the mouse IL-2 gene promoter and 5' flanking DNA linked to the bacterial gene encoding chloramphenicol acetyltransferase (CAT) and their transfection into cell lines

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Abbreviations: CAT, chloramphenicol acetyltransferase; Bt_2-cAMP, $N^6,O^{2'}$-dibutyryladenosine 3',5'-cyclic monophosphate; PGE_1, prostaglandin E_1; TCR, T-cell receptor; T_H cell, helper T cell; PMA, phorbol 12-myristate 13-acetate; IL-1, IL-2, etc., interleukin 1, interleukin 2, etc.

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have been described in detail elsewhere (18). Transfection of exponentially growing cells and assay of CAT activity were as described in detail (18).

RNA Extraction and Analysis. Cytoplasmic RNA was extracted by the method of Favaloro *et al.* (19) and analyzed as described (18).

cAMP Assay. Cells were resuspended at 1.5×10^6 cells per ml in complete medium containing various stimuli. After incubation for 15 min at 37°C, the reactions were terminated in liquid N_2. Ethanol was added to 65% and the tubes were vortex mixed until the cell pellet thawed. After clarification, supernatants were transferred to fresh tubes, dried in a vacuum centrifuge, and stored at −20°C until assayed. Assays were performed on extracts from 2×10^4 to 1.5×10^5 cell equivalents using a dual-range radioimmunoassay kit (Amersham). All samples and standards were acetylated according to the manufacturer's instructions prior to being assayed. In the absence of competition, 60–65% of the total tracer was precipitated and 2 fmol of competitor could readily be detected.

RESULTS

TCR signaling induces IL-2 expression by the inositol phospholipid pathway, using elevated intracellular Ca^{2+} and protein kinase C activation as dominant mediators (20). Agonists that raise levels of intracellular cAMP can sharply antagonize IL-2 induction (21–23). One proposed mechanism for the inhibitory effect of cAMP is that it acts to uncouple the TCR from the pathway generating inositol phospholipid breakdown products (24, 25). Here we show that it can also block IL-2 induction when saturating Ca^{2+} and protein kinase C signals are provided. Fig. 1 shows that even in the presence of optimal concentrations of PMA and A23187, which bypass the requirement for TCR coupling to phospholipase C, IL-2 RNA induction can be blocked in both EL4.E1 (Fig. 1*A*) and Jurkat (Fig. 1*B*) IL-2-producer cells by agents that increase intracellular cAMP. Forskolin (Fig. 1*A*, lane 7, and *B*, lane 5)

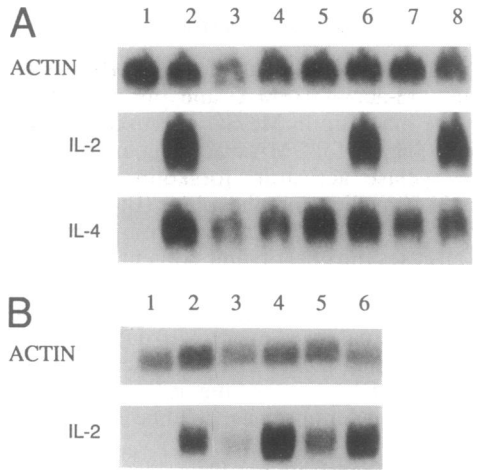

FIG. 1. Effect of drugs on IL-2 mRNA induction: Increased intracellular cAMP decreases IL-2 expression but not IL-4 expression. These gel blots of cytoplasmic RNA show the effects of increased intracellular cAMP on lymphokine mRNA levels. For each panel, the same filter was hybridized sequentially with probes indicated on the left. (*A*) EL4.E1 cells stimulated in the presence of various cAMP agonists. Lanes: 1, unstimulated cells; 2–8, cell stimulated 5 hr with PMA plus A23187. Other additives were as follows. Lanes: 2, no additives; 3, 10 μM PGE_1; 4, 1 μM PGE_1; 5, 1 mM Bt_2cAMP; 6, 0.1 mM Bt_2cAMP; 7, 10 μM forskolin; and 8, 1 μM forskolin. (*B*) Jurkat cells. Lanes: 1 and 2, as in *A*; 3, 1 mM Bt_2cAMP; 4, 0.1 mM Bt_2cAMP; 5, 10 μM forskolin; 6, 1 μM forskolin.

and Bt_2cAMP (Fig. 1*A*, lane 5, and *B*, lane 3) inhibited responses of both cell lines in a dose-dependent manner, and PGE_1 (Fig. 1*A*, lanes 3 and 4), which elevates cAMP levels more indirectly, also inhibited IL-2 induction in the EL4.E1 line. Although Jurkat and EL4.E1 are malignant cell lines, they differ in their minimal activation requirements for IL-2 expression and clearly represent derivatives of T cells at different stages of development (26–28). Thus, the sensitivity to inhibition by elevated cAMP levels seems to be characteristic of the IL-2 induction pathway *per se* and not a peculiarity of signal generation in a given cell type.

IL-4 expression can also be induced in response to A23187 and PMA, and two cell lines programmed to express IL-4 are analyzed in Fig. 2. Fig. 2 *Left* shows the responses of D10.G4.1 cells, a nontransformed T_H2 line that requires periodic restimulation with antigen (15), and Fig. 2 *Right* shows 32Dcl5, an IL-3-dependent premast cell line (29) that also expresses IL-4 upon stimulation (30, 31). As shown in Fig. 2, IL-4 induction in both lines was completely insensitive to the effects of forskolin or PGE_1 at doses (10 μM) that sharply inhibited accumulation of steady-state IL-2 RNA in EL4.E1 cells. Thus, induction of lymphokine gene expression by Ca^{2+} and protein kinase C is not universally inhibited by cAMP.

The cAMP insensitivity of IL-4 RNA induction was demonstrable not only in the D10.G4.1 and 32Dcl5 cells but also in EL4.E1 cells themselves. These tumor cells, unlike most long-term T-cell lines, express both IL-2 and IL-4 upon induction (Fig. 1*A*, lane 2). In the presence of cAMP agonists, even in the absence of any detectable IL-2 RNA, the IL-4 RNA levels induced in these cells (relative to actin RNA) were unchanged (Fig. 1*A*, lanes 3, 4, 5, and 7). Thus, even within the same cells, expression of IL-2 and IL-4 RNA appears to be regulated by distinct gene-specific pathways, only one of which is inhibited by cAMP.

IL-2 RNA accumulation is controlled both at the level of transcriptional induction and at the level of RNA stabilization (32, 33). To assess whether cAMP inhibits at the transcriptional level, we utilized a series of chimeric DNA constructs that we have derived (18) in which various extents of murine IL-2 regulatory sequence drive expression of a bacterial CAT gene. In these constructs, the sequences that target natural IL-2 RNA for degradation are deleted, and the level of CAT enzyme produced predominantly reflects promoter activity.

Fig. 3 shows the effects of cAMP elevation on levels of CAT activity expressed in EL4.E1 and Jurkat cells transiently transfected with these constructs, with pRSV–CAT as a control. In all cases, the cells were stimulated after transfection, as described (18), and the figure shows the activity of each construct after stimulation in the presence of the indicated drugs as a fraction of its activity after stimulation in the

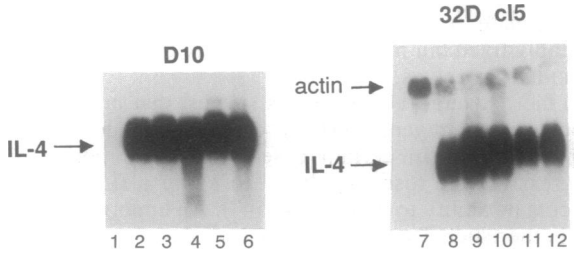

FIG. 2. Insensitivity of IL-4 induction to cAMP regardless of cell type: 32Dcl5 (lanes 1–6) and D10.G4.1 (lanes 7–12). Cytoplasmic RNA was separated electrophoretically and hybridized with the indicated probes as in Fig. 1. Cells were left unstimulated (lanes 1 and 7) or were stimulated for 5 hr with PMA plus A23187 alone (lanes 2 and 8) or in the presence of 10 μM (lanes 3 and 9) or 1 μM (lanes 4 and 10) forskolin and 10 μM (lanes 5 and 11) or 1 μM (lanes 6 and 12) PGE_1.

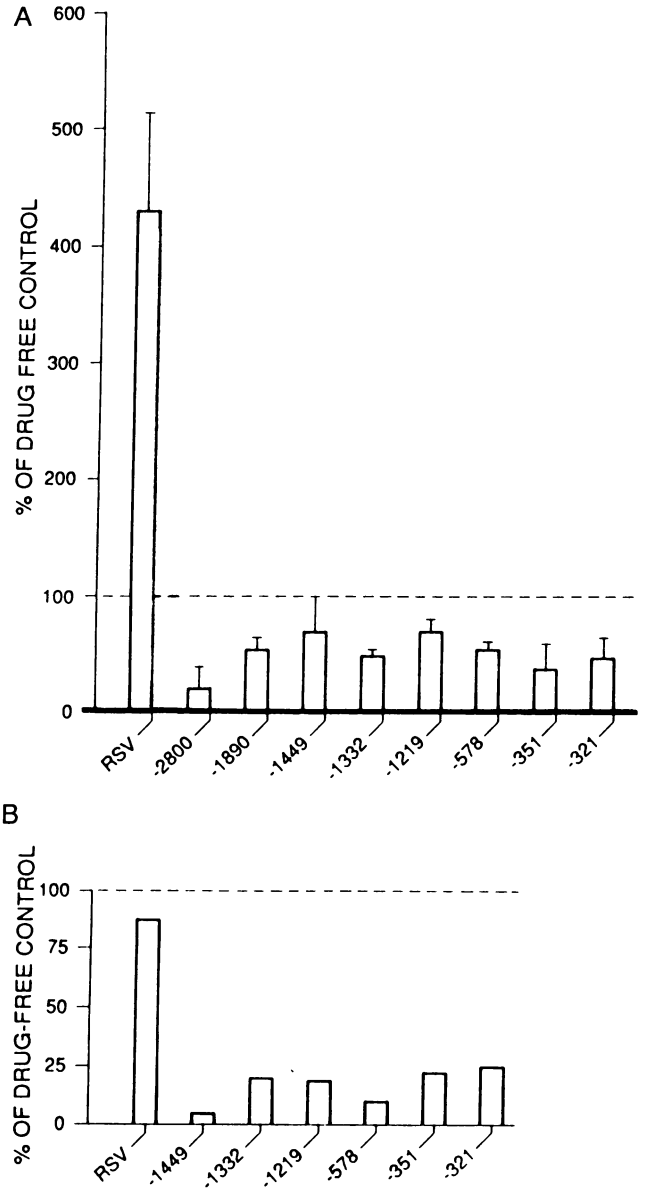

FIG. 3. Increased intracellular cAMP depresses expression of CAT from the IL-2 promoter in a promoter-specific way. (*A*) EL4.E1 cells transfected with the indicated plasmids were stimulated with PMA plus A23187 for 18 hr in the presence and absence of 10 μM forskolin. For each plasmid results were calculated as [(CAT activity with drug)/(CAT activity without drug)] × 100. Results are given as mean ± range or SEM of two or three experiments. Similar results were obtained with a treatment of 1 mM PGE_1 (data not shown). (*B*) Effect of 1 mM Bt_2cAMP on IL-2–CAT expression in Jurkat cells relative to drug-free controls. Results shown are from a single experiment. RSV, Rous sarcoma virus.

absence of the drug. Expression of all the pIL-2–CAT plasmids was suppressed by 30–80% in the presence of cAMP agonists, relative to their drug-free controls. Similar results in EL4.E1 cells were achieved with 1 μM PGE_1 (data not shown). At the same time, however, pRSV–CAT expression, which is also up-regulated by PMA and A23187, was minimally inhibited in Jurkat cells and actually increased an additional 4-fold in EL4.E1 cells under these conditions. Thus, the suppressive effects of cAMP on the pIL-2–CAT plasmids are not attributable to a generalized down-regulation of transcription, translation, or activation pathways. Instead, the same intracellular signal in the same T-cell population gives different effects on transcriptional induction, depending on the inducible gene being studied. Thus, at least one component of the cAMP effect on IL-2 induction affects transcriptional activity of the IL-2 gene in a promoter-specific manner.

Although cAMP agonists only partially inhibited IL-2–CAT expression, the inhibition was most pronounced at early times after induction. The greater inhibitory effect seen at 18 hr in the experiment shown with Jurkat (>75% inhibition) as compared to EL4.E1 (≈50%) reflected the slower kinetics of IL-2–CAT induction in Jurkat cells (unpublished observations). When extracts of stimulated EL4.E1 cells were assayed at 5 hr rather than 18 hr, 10 μM forskolin also caused more severe inhibition (≥70%; also, P. M. White and E.V.R., unpublished results). The initial kinetics of CAT product accumulation are more likely to be dominated by relative transcription rates *per se* and less by effects on transcript stability and translational efficiency than later plateau values. The greater inhibition at early times thus supports the interpretation that cAMP in fact inhibits transcription from the IL-2 promoter.

Other mechanisms might contribute to the effect of cAMP on IL-2 accumulation. The degree of inhibition of IL-2–CAT expression by cAMP was consistently less severe than the inhibition of endogenous IL-2 RNA accumulation. However, as the kinetics of IL-2–CAT induction in EL4.E1 cells are slower than those of IL-2 mRNA induction, single time-point measurements of their accumulated levels may not be directly comparable. Our results could suggest either that the effect of cAMP is transient or that it exerts some effects on IL-2 RNA at the post-transcriptional level.

To assess the biological significance of increased intracellular cAMP, we examined several T-cell populations that selectively produce IL-2 or IL-4. In addition to the T_H2 lines D10.G4.1 and CDC-25, we also examined a cloned T_H1 line, A.E7 (16). When assayed 7 days after stimulation with antigen, D10 cells contained 3 times more cAMP on a per-cell basis than A.E7 cells (Table 1). This difference in baseline cAMP levels was also seen 4 days after antigenic stimulation when both lines were actively proliferating. Another T_H2 line, CDC-25, contained even higher levels of cAMP. Thus, A.E7 cells maintain a low level of cAMP that is independent of their proliferative status.

The level of cAMP in these lines did not change significantly after stimulation with PMA plus A23187 or anti-CD3 plus IL-1. It therefore appears that the stable response phenotypes of these cloned lines are correlated with stably maintained differences between their levels of cAMP. Because cell lines may not be representative of normal cells, we also examined cAMP levels in $CD4^+$ splenic T cells. This laboratory has shown (3) that almost 100% of these cells synthesize IL-2 mRNA in response to PMA plus A23187. These cells have also been shown to secrete little, if any, IL-4 (5, 34). As shown in Table 1, freshly isolated $CD4^+$ T cells have resting levels of cAMP that are comparable to or slightly lower than those of A.E7 cells. Intriguingly, they even manifest a poor response to forskolin at this time. After several days of culture in the presence of concanavalin A and IL-4, many $CD4^+$ T cells acquire the ability to secrete IL-4 (35). These cells still maintained a relatively low level of cAMP that did not change when stimulated with PMA plus A23187 or anti-CD3 plus IL-1. However, they had acquired the ability to respond well to forskolin, a property that was evident by day 2 of culture. It is therefore possible that these cells could increase their intracellular pools of cAMP significantly more than fresh $CD4^+$ cells if they were triggered appropriately (see below). Thus, while the details of any such developmental changes remain to be explored, the results presented in Table 1 support a correlation between high cAMP levels and preferential production of IL-4 relative to IL-2.

Table 1. cAMP levels in IL-2 producer and nonproducer T-cell populations

Cell	Time of assay	Stimulation conditions	cAMP, fmol per 10^6 cells Exp. 1	Exp. 2
D10.G4.1	Day 4	–		700
	Day 7	–	507	547
	Day 7	PMA/A23187	700	547
	Day 7	Forskolin	1633	1800
	Day 7	CD3/IL-1	653	360
CDC-25	Days 3–5	–	3500	4800
A.E7	Day 4	–		160
	Day 7	–	160	190
	Day 7	PMA/A23187	200	147
	Day 7	Forskolin	2560	733
	Day 7	CD3/IL-1	230	147
$CD4^+$ spleen*	Day 0	–	113	87
	Day 0	PMA/A23187	93	
	Day 0	Forskolin	600	767
	Day 0	CD3/IL-1	93	
	Day 2	–		180
	Day 2	Forskolin		4100
	Day 4	–	160	
	Day 4	PMA/A23187	160	
	Day 4	Forskolin	4267	
	Day 4	CD3/IL-1	173	

D10.G4.1, CDC-25, and A.E7 cells were stimulated with antigen- and mitomycin C-treated spleen cells on day 0. Four and 7 days later cells were harvested and assayed for cAMP after no treatment (–) or after 15 min at 37°C with the indicated stimuli. In experiment 1, healthy CDC-25 cells were assayed 24 hr after thawing. Spleen cells were depleted of B cells by "panning" on goat anti-mouse immunoglobulin-coated plates and $CD8^+$ T cells were eliminated by two rounds of complement-mediated lysis (day 0). Day 2 spleen cells were grown in the presence of concanavalin A (3 μg/ml) and PMA (3 ng/ml). Day 4 cells received concanavalin A (2 μg/ml) on day 0. On day 1 the cells received 5% (vol/vol) EL4-conditioned medium and recombinant IL-4 (20 units/ml). Cells were stimulated with PMA and A23187 at concentrations of 10 ng/ml and 37 ng/ml, respectively. Anti-CD3 (ε) antibody 145-2C11 was used to coat polystyrene tubes and recombinant human IL-1α was used at a final concentration of 100 units/ml. Forskolin was used at 10 μM.

*Day 0 cells were >95% $CD4^+$ T cells in experiment 1 and were contaminated with 19% B cells in experiment 2. Day 4 cells were from a different starting population, and at the time of harvest were >75% T cells of which >99% were $CD4^+$.

DISCUSSION

We have shown that, in a variety of clonal cell lines, treatments that elevate intracellular cAMP levels can abort IL-2 RNA induction while leaving IL-4 induction, by the same pharmacological stimuli, unaffected. A particularly dramatic example is provided by the EL4.E1 line, where induction of the two lymphokines can be differentially affected within the same cells. Under the conditions used here, 80–100% of these EL4.E1 cells accumulate IL-2 RNA in the absence of cAMP (unpublished results). Thus, it is likely that the same cells that appear cAMP-resistant for IL-4 RNA induction are also cAMP-sensitive for IL-2 RNA induction. Although both lymphokine genes can be induced by Ca^{2+} ionophores and phorbol esters as well as TCR-mediated signaling, their induction pathways are in fact distinct.

The effect of cAMP on IL-2 accumulation at least includes a component of transcriptional inhibition. In spite of the various degrees of inhibition observed under different conditions, all of the nested series of 5′ IL-2–CAT constructs tested here were inhibited by cAMP to the same extent within experimental error. Thus, neither the weak negative regulatory sites nor the positive regulatory regions that we have identified (18) upstream of position −321 are likely to be specific targets of the cAMP pathway blocking IL-2 induction. This conclusion is in agreement with the absence of identifiable "cAMP response elements" in the entire 2800 base pairs of IL-2 5′ flanking sequence (18). However, more recent data suggest that cAMP inhibition involves a complex of positive and negative factors acting at distinct sites (ref. 36; P. M. White and E.V.R., unpublished results).

The differential inhibition of IL-2 by cAMP may be more than a pharmacological curiosity *in vitro*. Accumulating evidence suggests that T_H1 and T_H2 type memory cells may be divergent products of a single $CD4^+$ lineage, with at least one intermediate cell type capable of expressing both IL-2 and IL-4. With elevated cAMP levels, such cells could behave indistinguishably from T_H2 cells. It is, therefore, provocative that we find that D10.G4.1 and CDC-25 cells sustain levels of cAMP throughout their stimulation cycle that are consistently higher than those of A.E7 cells. The high levels of cAMP found in the T_H2 cells also distinguish them sharply from freshly isolated thymocytes (data not shown) or splenocytes, all of which make IL-2 much more readily than IL-4 in our hands (T.J.N., Dan Chen, and E.V.R., unpublished results; ref. 3).

The levels of cAMP in D10.G4.1 cells are unlikely to be sufficiently high to explain the complete block to IL-2 induction in these cells; the similar concentrations induced by forskolin treatment in fresh spleen cells only reduce IL-2 secretion by 3- to 5-fold (Patricia M. White and E.V.R., unpublished results). Nevertheless, cAMP at 500–700 fmol per 10^6 cells may participate in "handicapping" the induction pathway for IL-2. The cAMP concentration we find in CDC-25 cells is more likely to be prohibitive in itself. It is also intriguing to speculate that these elevated cAMP levels are a relic of a more acute mechanism that originally drove the transition of some bifunctional precursor cells into the T_H2 functional type. Further work will be needed to test this possibility, but our results with splenic $CD4^+$ cells may provide a starting point. Fresh resting splenic $CD4^+$ cells displayed only a modest ability to elevate cAMP in response to forskolin, suggesting that their adenylate cyclase activity is relatively low and/or their phosphodiesterase activity is high. Two to 4 days after activation, the main difference observed was a sharp increase in the magnitude of their forskolin response. Thus, not their level of cAMP *per se* but their capacity to elevate cAMP in response to potential stimuli was significantly enhanced. Such preactivated cells could be much more susceptible than fresh resting $CD4^+$ cells to environmental stimuli that, unlike the artificial ones tested here, do activate adenylate cyclase. Thus, although repeated stimulation need not shut off IL-2 inducibility directly, through use of cAMP, repeated stimulation could make loss of IL-2 inducibility much more probable.

A report by Groux *et al.* (37) reinforces the possibility that elevation of cAMP may be especially implicated in signaling in memory T_H2 cells. In humans, CD29 expression has long been used as a specific marker of memory T cells, in particular those that provide help most efficiently for B cells (38–41). B-cell help commonly reflects the production of IL-4 and IL-5 more than that of IL-2 (42). Furthermore, CD29, or a close relative, appears to act as a Peyer's patch homing receptor (43) [i.e., a component of the mechanism that selectively concentrates those helper cells *in vivo* whose effects are most T_H2-like]. It is thus particularly interesting that CD29 molecules can directly transduce signals leading to sustained cAMP accumulation, with concomitant decreases in the inducibility of IL-2 (37). The memory-cell marker may thus participate in directing the memory-cell-specific response.

Of course, the ability of cAMP agonists to mimic the "T_H0" to "T_H2" transition in one step does not prove that cAMP is the primary regulator of T-cell functional type. It does, however, suggest a general theme for the programming of T-cell effector subclasses. Use of common second messengers like cAMP in a critical balance with other activation signals to dictate not only the magnitude but also the nature of a response provides a highly plastic, reversible way of controlling functional identity. It may be that such mechanisms play a dominant role in the differentiation of the highly diverse family of hematopoietic cell types.

Note. After this manuscript was submitted, Muñoz *et al.* (44) reported results complementary to ours and confirmed that forskolin and cholera toxin block PMA plus ionomycin induction of IL-2 in a T_H1 line.

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